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14. ABSTRACT
Ovarian cancer (OC) is the deadliest of all gynecological cancers, with five year survival rates of <45%. One critical feature of the disease is that two-thirds of the women diagnosed have advanced disease, and the five year survival rate of this group is <30%. This project outlines the development of a recombinant version of a member of a class of proteins known as disintegrins as an innovative imaging and diagnostic agent for ovarian cancer (OC). Vicrostatin (VN) is a recombinant protein based on the venom disintegrin contortrostatin (CN), which has shown impressive antitumor and antiangiogenic activities in models of human ovarian cancer. OC cells have been shown to display integrins $\alpha v\beta 5$ and $\alpha 5\beta 1$, and the antitumor activity of CN, and demonstrated for VN, is based on the high affinity interaction between the disintegrin and these integrins. Thus far we have developed and shown that we have a robust and viable system for the production of VN and that the protein produced displays a high affinity for integrins displayed on ovarian cancer cells. In ongoing experiments we are evaluating the imaging potential for VN to be used for both evaluation of treatment and diagnosis of OC. The high affinity of VN for the integrins found on OC cells make for an excellent candidate for improvement of OC diagnosis and therapy.

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Annual Progress Report

Introduction

The project entitled “Development of a Multifaceted Ovarian Cancer Imaging Agent” will cover the period of time from April 1 2007-March 31, 2010, this report covers the second annual period of the project April 1, 2008–March 31, 2009. The project focuses on the development of a production method for a recombinant disintegrin vicrostatin (VN), whose structure is based on the snake venom disintegrin contortrostatin (CN). The protein is delivered via intra-peritoneal injection in a liposomal formulation. PET imaging radiotracers can be covalently attached to VN and used as an imaging and diagnostic agent in ovarian cancer (OC). In the past year we utilized our expression system, described in last year’s report, to produce VN for use as an imaging agent. In addition we have further evaluated and quantitatively determined the integrin affinity of VN for a select group of integrins thought to be important in ovarian cancer. Also, we evaluated the circulatory half-life of both VN and liposomal encapsulated VN (LVN). By using a short peptide sequence that was derived from the structure of a disintegrin, we have shown that there is specific labeling and uptake of the fluorescently labeled peptide by an orthotopic, xenograft ovarian tumor. Finally, we are progressing on the development of VN as an OC imaging agent and have evaluated methods for radionuclide attachment to VN and made initial attempts to use the agent in PET imaging.

Summary of Progress on Specific Aims

During this year we were able to make significant progress toward successful completion of the goals and milestones of this project. Building on work from the previous year we were able to begin to develop PET imaging agents based on the disintegrins we have been studying. We determined the affinity of the disintegrin for several integrins, showed that LVN formulation increases circulatory half-life of the encapsulated disintegrin compared to the naked protein, demonstrated that integrin ligation is possible under *in vivo* conditions and finally began the formulation and production of VN as a PET imaging agent. Progress in these studies has lead to significant advances toward our final two milestones. Our Specific Aims and Milestones for this project are:

Specific Aim 1: Prepare VN, a recombinant disintegrin with proven *in vivo* antiangiogenic activity (**Milestone 1**, **completed Year 1**), and produce a liposomal formulation (LVN) with stability characteristics appropriate for clinical application (**Milestone 2**, **completed Year 1**).

Specific Aim 2: Demonstrate imaging potential and biological efficacy of a LVN formulation in a mouse model of ovarian cancer (Milestone 3, in progress).

Specific Aim 3: Evaluate the use of VN as a novel tumor imaging agent both for diagnostic use and for evaluation of tumor suppression following treatment (Milestone 4, in progress).

Body

Binding to integrins on cell surfaces: In the previous year’s progress report we showed that recombinant VN binds with different affinities to a panel of human ovarian cancer cell lines dependent on the integrin display status of the individual cell line. To quantitatively assess the binding affinities of VN with soluble functional integrins, two approaches were evaluated. First the disintegrins were immobilized on the surface of a BiaCore surface Plasmon resonance chip. The soluble integrin was then allowed to interact with the immobilized disintegrin. This approach was unsuccessful with VN, presumably because the integrins had limited access to VN. This was confirmed by flowing an anti-VN antibody across the surface of the chip and the antibody also displayed limited binding. It is believed that the disintegrin interaction with the carboxymethyl cellulose surface causes VN to be buried and unavailable for binding. In an attempt to solve this issue fluorescence polarization (FP) was used to determine binding kinetics. In this method, differing concentrations of functional integrin were incubated with a constant amount of FITC labeled VN. As VN is a small molecule it rapidly depolarizes the excitation light. Upon binding to the large integrin, the fluorescent tag on VN tumbles in solution at a slower rate resulting in increased levels of polarization. The measured FP value is a weighted average of FP values of the bound and free fluorescent VN and is therefore a direct measure of the fraction bound. Data generated in these experiments can be analyzed like standard radioligand binding, and kinetics of binding can be determined as with Scatchard analysis using a non-linear curve fit. From this set of experiments we determined the dissociation constants for VN and CN with integrins $\alpha v\beta 3$, $\alpha 5\beta 1$ and $\alpha v\beta 5$ (**Table 1**). Recombinant VN was purposely designed with a carboxy-terminal extension, which was expected to enhance affinity for α5β1. This was confirmed as CN and VN exhibit nearly identical affinities for αvβ3 and similar affinity for αvβ5, while there is an order of magnitude difference in the Kd values for binding to α5β1 when comparing VN (higher affinity binding) to CN. In the evaluation of the dissociation constants of VN for integrins αvβ3, αvβ5 and α5β1 (integrins that are over expressed on ovarian cancer cell lines) we found that the carboxy-terminal modification in VN does impart a higher affinity for α5β1, as designed. The results of these studies support our hypothesis that the promiscuous nature of integrin binding by VN allows for broad targeting toward ovarian cancer.

Table 1 Dissociation constants for interactions of VN and CN with Soluble Integrins

Disintegrin	Integrin Kd		
	αvβ3	α5β1	αvβ5
CN	6.6nM	191.3nM	19.5nM
VN	7.4nM	15.2nM	41.2nM

Values calculated through fluorescence polarization measurements following steady state binding

Determination of Circulatory half-life of LVN: Previously we had determined the circulatory half-life of CN and liposomal CN. We repeated these studies for VN and liposomal VN (LVN). To carry out this study blood samples were taken 0.5, 1, 3, 6, 18, 24, 48 and 72 hours following i.v. administration of ^{125}I-VN or L-^{125}I-VN. Gamma counting of collected blood samples revealed that there was a rapid decrease to <.1% of the administered counts in the blood at 6 hours after i.v. injection of ^{125}I-VN. However, in animals given L-^{125}I-VN, the percentage of total injected counts in the blood drops to a level of 63% of the injected counts 6 hours post-injection and gradually decreases over the following 66 hours. By plotting the decrease in radioactivity in blood over time following i.v. administration in tumor-free mice, we observed a circulatory half-life of 0.4 h for ^{125}I-VN and 20.4 h for L-^{125}I-VN. Thus, encapsulation of VN in liposomes not only protects the protein but also maintains it in the circulation for a much longer period of time than native unencapsulated VN, enabling more effective access to the tumor.

Labeling VN for use as a PET Imaging Agent: For use as a PET imaging agent in ovarian cancer VN needs to be complexed with an appropriate PET radionuclide. In this instance the radionuclide utilized was ^{64}Cu, selected due to its relatively long (for a PET agent) half life, 12.7hrs, which makes it more suitable for larger peptide or protein based PET imaging, and more effective for conjugation to the macrocylic chelator 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA), a heterobifunctional crosslinker that can be attached to Lys residues in proteins. To create a VN DOTA conjugate DOTA is first activated by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and N-hydroxysulfonosuccinimide (SNHS) at pH 5.5 for 30 min with a molar ratio of DOTA:EDC:SNHS = 10:5:4. The DOTA-OSSu reaction mixture (15 µmol, calculated on the basis of SNHS) was cooled to 4°C and added to VN (3:1 molar ratio) dissolved in 500 µL of water. The reaction mixture was adjusted to pH 8.5 with 0.1N of NaOH and was allowed to incubate overnight at 4°C. The DOTA-coupled VN was purified by semipreparative HPLC. The peak containing the conjugate was collected, lyophilized, and dissolved in water (2 mg/mL) for use in radiolabeling reactions. For ^{64}Cu labeling of the VN:DOTA conjugate 20 µL of $^{64}CuCl_2$ (74 MBq in 0.1N HCl) was diluted in 400 µL of 0.1 mol/L sodium acetate buffer (pH 6.5) and added to the DOTA-VN solution (a 1 mg/mL solution of peptide was made and separated into aliquots; 80 µg of DOTA-VN per 37 MBq of ^{64}Cu were used for the labeling). The reaction mixture was incubated for 1 h at 40°C. ^{64}Cu-DOTA-VN was then purified by semipreparative HPLC, and the radioactive peak containing the desired product was collected. After removal of the solvent by rotary evaporation, the residue was reconstituted in phosphate-buffered saline for *in vivo* animal experiments. The labeling yield of ^{64}Cu-DOTA-VN is 74% based on the HPLC profile. Current studies are ongoing to evaluate the retention of bioactivity of the labeled VN.

Binding of an integrin targeted peptide to an orthotopic xenograft ovarian tumor: In these studies we established an orthotopic xenograft ovarian tumor through surgical exposure of the ovary and direct injection of the ovarian cancer cells ($1x10^6$ cells in 50µl) into the stroma of the ovary. The tumors were allowed to grow untreated for 28 days. At this time a disintegrin based, integrin targeted peptide ligand was injected systemically by intravenous injection. The FITC labeled cyclic peptide contains the sequence CTRKKHDNAQC, cyclized through the Cys sidechains, and is based on the putative integrin binding loop of the disintegrin domain of Jararhagin, a metallopreoteinase with a disintegrin domain isolated from *Bothrops jararaca*, a venomous pit viper found in Brazil, Paraguay and northern Argentina. When the peptide is injected systemically (10µg/mouse) and the ovary is surgically exposed and imaged (Fluorescence Mode, Xenogen IVIS100 System), **Figure 1** shows that there is a high level of fluorescence associated with the tumor tissue surrounding the ovary. Conversely the tissue surrounding the control non-tumor bearing ovary shows little fluorescence indicating a specific binding to the tumor cells by the fluorescently labeled integrin ligand. Studies are in progress to repeat this experiment with FITC-VN.

Key Research Accomplishments

- Determined the enhanced affinity of the recombinant disintegrin for an integrin overexpressed on ovarian tumors
- Evaluated the circulatory half-life of VN versus LVN
- Determined a method to radiolabel VN (and CN) with PET radionuclides
- Evaluated the ability and specificity of an integrin targeted peptide ligand to bind to an orthotopic xenograft ovarian tumor

Reportable Outcomes

A manuscript is in preparation describing the production of VN and assays of VN’s activity

Conclusion

LVN is a novel liposomal formulation of a disintegrin engineered using standard recombinant techniques. The results from the Year I studies clearly show that LVN prepared by a commercially viable technique retains integrin binding and antiangiogenic activity equivalent to the laboratory prepared material. In the second year of this project we have been successful in determining the Kd of VN for integrins important to OC. We have shown that integrins can be specifically labeled *in vivo*, and finally we have begun development of the methods necessary for use of VN as PET imaging agent.

References

None

Appendices

Figure 1

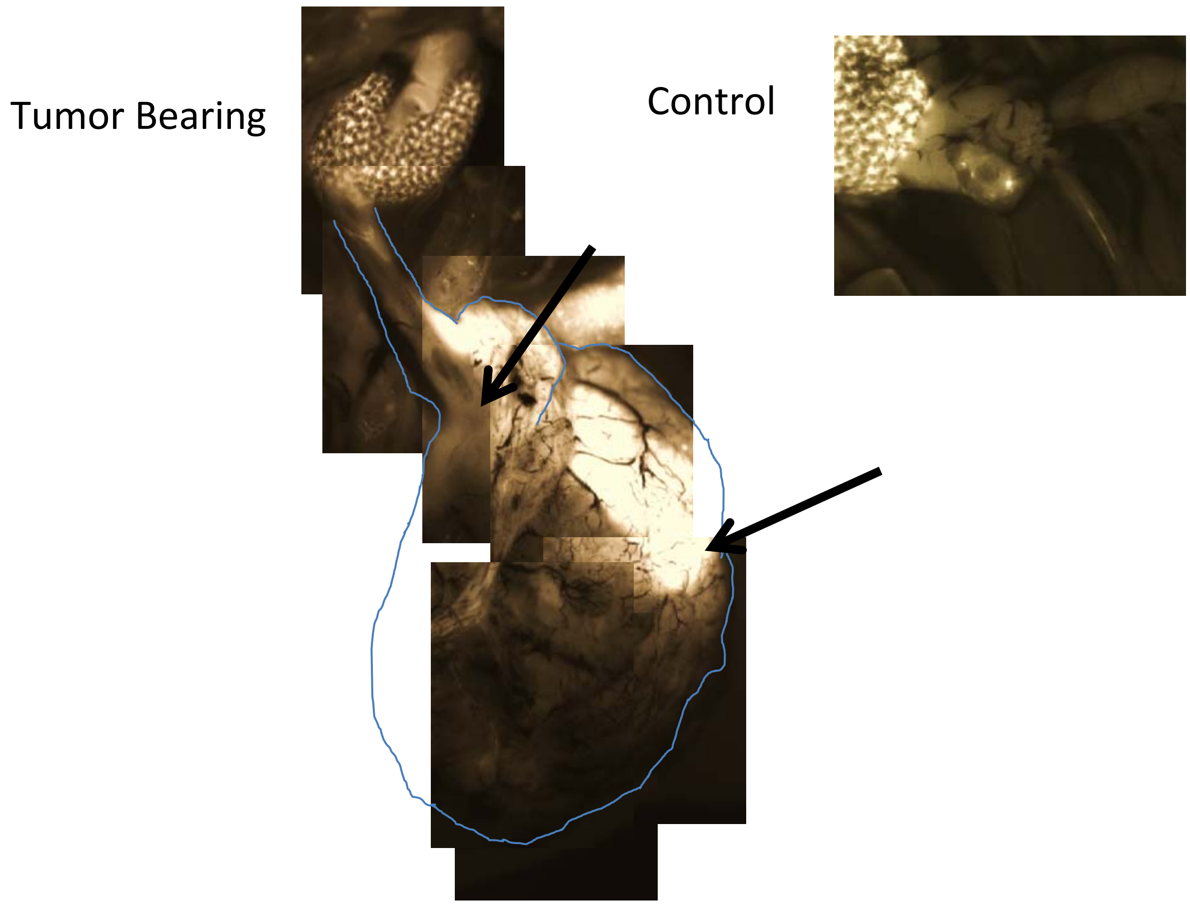

Figure 1 FITC labeled disintegrin based peptide specifically binds to integrins on an orthotopic xenograft ovarian tumor. OVCAR-3 cells were implanted orthotopically following surgical exposure of the ovary of a balb/c/nu/nu mouse. The tumor was allowed to grow untreated for 28 days. At this time a FITC labeled peptide, CTRKKHDNAQC cyclized through the Cys sidechains, was delivered IV. Thirty minutes post peptide injection the ovary is again surgically exposed and a fluorescent and optical image of the ovary and surrounding tissue, normal and tumor, were obtained. A high level of fluorescence is observed with in the tumor while little fluorescence is observed in the tissue surrounding the control ovary. Black arrows indicate areas of fluorescence. The blue line represents the margins of the tumor and the stitched image in the tumor sample to show the large tumor is due to the limited area in the photographic field of the Xenogen instrument.